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Tumorigenesis

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Introduction

The overall goal of this project is to understand the role of up-stream I κ B kinases (IKK) and NF- κ B "survival signaling" pathway in tumorigenesis in prostate. During first two years we found that NF- κ B was constitutively activated in human androgen-independent PC cell lines due to the constitutive activation of IKK α/β kinases. We also found that IKK ϵ was strongly expressed only in androgen-independent PC cells and may be involved in the NF- κ B induction in PC cells through a positive feedback loop. The results of our experiments also showed that in contrast to other tumor cell types with constitutively activated NF- κ B, PC cells independently on the basal level of NF- κ B, are highly sensitive to NF- κ B activation by different standard NF- κ B inducers. We have generated @ 100 clones of different PC cells transfected with d.n. mutants of different IKKs. During the current funding period my laboratory has moved from AMC Cancer Center in Denver to Northwestern University in Chicago. During first several months of transition period I had to rebuild my laboratory, transfer awards to NU, hire new postdoctoral fellows and technicians, prepare new IRB and IACUC protocols. I have accomplished all these goals successfully. In addition, I became a member of NU Cancer Center, the member of NU prostate SPORE program, and a member/mentor of NU integrated graduate program. My laboratory also participates in summer student training program, and this year one of the summer students will be involved in the studies focused on the role of NF- κ B in prostate tumorigenesis funded by DOD. In spite of transition period we continued to work actively on the project. The results of our studies have been presented at national and local meetings, one manuscript was submitted. The following describes progress made in this year.

Body

During current year my laboratory moved from AMC Cancer Center in Denver to Northwestern University in Chicago. As a result of my move from Denver to Chicago and grant transfer I needed more time to complete the proposed work, so I requested the grant extension for one year (till 06.01.2005) at no additional cost. In spite of the move and the transition period that was needed to organize the laboratory and hiring personnel, we continued to work actively on the project.

Specifically we focused on the task 4 pertinent to the part of the Specific Aim 1, and studied the expression of NF- κ B and IKK proteins in prostate tissues. We performed immunostaining of more than 60 formalin-fixed paraffin-embedded samples (including tissue microarrays and individual sections) of BPH, high grade PIN, and PCs using multiple antibodies against NF- κ B and IKK proteins: p65 (two different Abs from Abcam and Santa Cruz), phosphorylated p65 (Cell Signaling), p50 (Santa Cruz), p52 (Santa Cruz), IKK α (two different Abs from Santa Cruz and Imgenix), IKK β (Imgenix), phosphorylated IKK α /IKK β (Cell Signaling), and IKK ϵ (four different Abs from Imgenix, Santa Cruz., Active Motif, Pro-Sci). We found that there was relatively modest increase of number of p65-positive nuclei in low grade and advanced PCs in comparison to BPH. There were no significant changes in the expression of p50 and

IKK β in PC in comparison to BPH. P52 expression was significantly higher in high grade PCs in comparison to BPH and low grade PCs. Importantly we found frequent nuclear localization of p52 in PCs. In some tumors p52 immunostaining pattern correlated with IKK α expression, and especially with the level of IKK α / β phosphorylation. The preferential increase in the expression of p50 (NF- κ B1) and p52 (NF- κ B2) proteins in many different tumor cell lines and in tumors has been previously reported (reviewed in Greten and Karin, 2004). It was important to study whether p52 expression in PCs correlates with IKK α expression and phosphorylated kinase because it was shown recently that activated (phosphorylated) IKK α is necessary for processing of p52 precursor p100 (Senftleben et al., 2001).

We also focused on the tasks 8 -10. We have previously generated clones of PC cells transfected with d.n. mutants of different IKKs, and in pilot experiments have assessed the effect of d.n. IKK mutants on NF- κ B activity. Unfortunately, in most of those transfected clones the expression of IKK β d.n. and IKK α d.n. mutants significantly decreased during first 10 passages. To overcome the transgene silencing problem we have undertaken an alternative approach and during the current funding period performed the experiments with highly specific chemical inhibitor of IKK β , PS1145 (Millenium Pharmaceuticals Inc.). We have developed a comprehensive picture of effects of PS1145 on prostate cells in vitro including its effect on NF- κ B activity, sensitivity of PC cells to apoptosis, and the effect on PC cell' invasion potential. We found that PS1145 inhibited constitutive and inducible NF- κ B activity in androgen-independent PC cells, and blocked the constitutive expression of cytokines, including IL6. PS1145 induced apoptosis in PC cells and also significantly increased their sensitivity to TNF- α -induced apoptosis through caspase dependent pathway. We found that preincubation with PS1145 inhibited the invasion activity of PC3 cells in invasion chamber assay in a dose-dependent manner. This work has prepared grounds for the future studies of the effect of IKK chemical inhibitors in preclinical trial on human PC xenografts in vivo. As was originally proposed we have started to generate inducible IKK α d.n. and IKK β d.n. constructs using retroviral Tet-On expression system (Clontech Laboratories).

Next year we will continue to evaluate the expression of NF- κ B and IKK proteins in prostate tissues using Western and Northern blotting. We will use inducible d.n. IKK-expressing PC clones to study the role of specific IKKs on PC cell growth in vitro and in vivo in nude mice.

Key Research Accomplishments

- ❖ A specific IKK β inhibitor, PS1145 dramatically diminished the amount of phosphorylated I κ B α and blocked basal NF- κ B activity in androgen-independent PC cell lines DU145 and PC3. PS1145 also blocked activation of NF- κ B by TNF- α and LPS.
- ❖ PS1145 inhibited constitutive expression of different κ B-responsive genes and blocked the constitutive expression of cytokines, including IL6 in PC cells
- ❖ PS1145 induced apoptosis in PC cells and also significantly increased their sensitivity to TNF- α -induced apoptosis through caspase-dependent pathway.
- ❖ We found that preincubation with PS1145 inhibited the invasion activity of PC3 cells in invasion chamber assay in a dose-dependent manner.
- ❖ The analysis of immunostaining of prostate tissues has revealed modest increase of number of p65-positive nuclei in advanced PCs in comparison to BPH. There were no significant changes in the expression of p50 and IKK β in PC in comparison to BPH. P52 expression was significantly higher in high grade PCs in comparison to BPH. Importantly we found frequent nuclear localization of p52 in PCs. In some tumors p52 immunostaining pattern correlated with IKK α expression, and especially with the level of IKK α / β phosphorylation.

Reportable outcomes

Manuscripts

1. Yemelyanov A., Gasparian A., P. Lindholm, Dang L., Pierce J., Budunova I., Effect of IKK-beta specific inhibitor PS1145 on NF-kappaB activity, apoptosis and invasion in prostate carcinoma cell lines. Submitted.

Abstracts presented at national meetings

1. Gasparian, A. V., Yao, Y. J., Slaga T.J. and Budunova, I. V. High sensitivity of prostate carcinoma cell lines to NF-kB induction. Proceedings of AACR, 44: 1451, 2003.
2. Yemelyanov, A., Yao, Y.J, and Budunova, I. IKKi is a component of the positive feedback loop involved in the constitutive activation of NF-kB in prostate carcinoma cells. Proceedings of AACR 44: 852, 2003.
3. Yemelyanov A., Gasparian A., Dang L. Pierce J., Budunova I. IKK-beta specific inhibitor PS1145 down-regulates NF-kappaB activity and induces apoptosis in prostate carcinoma cell lines. Keystone Symposium: NF-kB: biology and pathology. January 11-16, 2004, Snowbird, Utah, p. 59.

4. Yemelyanov A., Yao Y, and Budunova I. Possible role of IKKi in the constitutive activation of NF- κ B in prostate carcinoma cells. Keystone Symposium: NF- κ B: biology and pathology. January 11-16, 2004, Snowbird, Utah, p. 101.
5. Budunova I., Yemelyanov A., Gasparian A., Dang L., Pierce J. Effect of IKK-beta specific inhibitor PS1145 on NF-kappaB activity and apoptosis in prostate carcinoma cell lines. Proceedings of AACR 45, 2004 (abstract # 4572).

Abstracts presented at local meetings

1. Yemelyanov A., Gasparian A., Dang L. Pierce J., Budunova I. IKK-beta specific inhibitor PS1145 down-regulates NF-kappaB activity and induces apoptosis in prostate carcinoma cell lines.
Northwestern University, All Campus Research, March , 2004, Chicago. **First prize for research presentation.**

Seminar presentations by PI

1. Constitutive activation of NF- κ B in prostate carcinoma cells: possible role of feedback loop involving IKK ϵ . Department of Urology seminar program, Feinberg School of Medicine, Northwestern University, Chicago, September, 2003.

Conclusions

Our data suggest that blockage of NF- κ B activity in PC cells by specific IKK β inhibitor PS1145 results in significant sensitization of PC cells to apoptosis. PS1145 also inhibited PC cell invasion in invasion chamber assay. Immunostaining of prostate tissues with NF- κ B and IKK antibodies revealed that p52 (NF- κ B2) expression was significantly higher in high grade PCs in comparison to BPH and low grade PCs. We found frequent nuclear localization of p52 in PC that generally correlated with IKK α expression, and especially with the level of IKK α/β phosphorylation.

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3. Greten FR, Karin M. The IKK/NF-kappaB activation pathway-a target for prevention and treatment of cancer. Cancer Lett. 2004;206(2):193-199.

Appendices

1. Gasparian, A. V., Yao, Y. J., Slaga T.J. and Budunova, I. V. High sensitivity of prostate carcinoma cell lines to NF-kB induction. *Proceedings of AACR*, 44: 1451, 2003.
2. Yemelyanov, A., Yao, Y.J, and Budunova, I. IKKi is a component of the positive feedback loop involved in the constitutive activation of NF-kB in prostate carcinoma cells. *Proceedings of AACR 44*: 852, 2003.
3. Yemelyanov A., Gasparian A., Dang L. Pierce J., Budunova I. IKK-beta specific inhibitor PS1145 down-regulates NF-kappaB activity and induces apoptosis in prostate carcinoma cell lines. *Keystone Symposium: NF-kB: biology and pathology*. January 11-16, 2004, Snowbird, Utah, p. 59.
4. Yemelyanov A., Yao Y, and Budunova I. Possible role of IKKi in the constitutive activation of NF-kB in prostate carcinoma cells. *Keystone Symposium: NF-kB: biology and pathology*. January 11-16, 2004, Snowbird, Utah, p. 101.
5. Budunova I., Yemelyanov A., Gasparian A., Dang L., Pierce J. Effect of IKK-beta specific inhibitor PS1145 on NF-kappaB activity and apoptosis in prostate carcinoma cell lines. *Proceedings of AACR 45*, 2004 (abstract # 4572).

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Proceedings

#R4300 IKKi is a component of the positive feedback loop involved in the constitutive activation of NF- κ B in prostate carcinoma cells. Alexander Yemelyanov, Ya Juan Yao, and Irina V. Budunova. AMC Cancer Research Center, Denver, CO.

Our recent data and data by others indicate that NF-kappaB is constitutively activated in androgen-independent prostate carcinoma (PC) cells and prostate tumors, and that NF-kappaB activation promotes PC cell tumorigenicity, invasiveness and resistance to apoptosis. The important step in NF-kappaB activation is the phosphorylation of IkappaB inhibitor proteins by IKK kinases: IKKa, IKKb and IKK-related inducible kinase IKKi. IKKi is the only IKK whose activity is regulated by its expression. We found that IKKa and IKKb were uniformly expressed in primary prostate cells and PC cell lines. On contrast, IKKi was strongly expressed only in androgen-independent PC cells (PC3 and DU145) with high level of constitutively active NF-kappaB but not in androgen-dependent PC cell lines (LNCaP and MDA PCa 2b) and primary prostate epithelial cells. Immunostaining also revealed that IKKi was expressed in human prostate carcinomas. Treatment of PC cells with NF-kappaB inducers such as IL-1 and TNF-alpha resulted in a rapid induction of IKKi. Transient transfection of different PC cell lines with IKKi w.t. resulted in activation of kB.Luciferase reporter, whereas IKKi dominant negative (d.n.) mutant K38A suppressed basal NF-kappaB activity in PC cells. These data provide experimental evidence that IKKi could be involved in the regulation of NF-kappaB activity in PC cells through a positive feedback loop. Supported by DOD Prostate Cancer Research Program DAMD 17-01-1-0015 and University of Colorado prostate SPORE developmental program.

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High sensitivity of prostate carcinoma cell lines to NF-[kappa]B induction.

Alexander V. Gasparaian, Ya Juan Yao, Thomas J. Slaga, Irina V. Budunova, Institute of Carcinogenesis, N. Blokhin Cancer Research Center, Moscow, Russia; AMC Cancer Research Center, Denver, CO; AMC Cancer Research Center, Lakewood, CO.

One of the central mechanisms protecting cells from apoptotic death is mediated by NF-[kappa]B transcriptional factors that control function of numerous cell survival genes. Our recent data and data by others showed that NF-[kappa]B is constitutively activated in androgen-independent prostate carcinoma (PC) cells and prostate tumors, and that NF-[kappa]B activation promotes PC cells resistance to apoptosis induced by chemo-therapeutical compounds. The results of our experiments indicated that androgen-independent PC cells maintain the high level of NF-[kappa]B basal activity by employment of the mechanism similar to that for NF-[kappa]B activation by inducers such as cytokines. This includes constitutive IKK activation, phosphorylation and fast turnover of I[kappa]B[alpha] inhibitor in androgen-independent PC cells. To find whether the high basal level of NF-[kappa]B activity in PC cells affects further NF-[kappa]B induction, we analyzed the sensitivity of normal prostate epithelial cells and PC cell lines to the standard NF-[kappa]B inducers such as TNF-[alpha], TPA and LPS. The results of our experiments showed that in contrast to other tumor cell types with constitutively activated NF-[kappa]B, PC cells independently on the basal level of NF-[kappa]B, are highly sensitive to NF-[kappa]B activation. The lack of response of LNCaP cells to LPS and DU145 cells to TPA rather reflects the cell-specific changes in the upstream signaling than function of NF-[kappa]B transcription factor. Supported by DOD Prostate Cancer Research Program DAMD17-01-1-0015.

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Possible role of IKKi in the constitutive activation of NF-kappaB in prostate carcinoma cells

Alexander Yemelyanov, Ya Juan Yao, and Irina Budunova
Northwestern University, Chicago, IL 60611

Our recent data and data by others indicate that NF-kappaB is constitutively activated in androgen-independent prostate carcinoma (PC) cells and prostate tumors, and that NF-kappaB activation promotes PC cells' tumorigenicity, invasiveness and resistance to apoptosis. The important step in NF-kappaB activation is the phosphorylation of IkappaB inhibitor proteins by IKK kinases: IKKalpha, IKKbeta and IKK-related inducible kinase IKKi. IKKi is the only IKK whose activity is regulated by its expression. We found that IKKalpha and IKKbeta were uniformly expressed in primary prostate cells and PC cell lines. On contrast, IKKi was strongly expressed only in androgen-independent PC cells (PC3 and DU145) with high level of constitutively active NF-kappaB but not in androgen-dependent PC cell lines (LNCaP and MDA PCa 2b) and primary prostate epithelial cells. Immunostaining also revealed that IKKi was expressed in human prostate carcinomas. Treatment of PC cells with NF-kappaB inducers such as IL-1 alpha and TNF-alpha resulted in a rapid induction of IKKi. Consistent with this, down-regulation of NF-kappaB activity by proteasome inhibitor MG132 attenuated induction of IKKi expression by NF-kappaB inducers. Transient transfection of different PC cell lines with IKKi w.t. resulted in activation of kappaB.Luciferase reporter, whereas IKKi dominant negative (d.n.) mutant K38A suppressed basal NF-kappaB activity in PC cells. These data provide experimental evidence that IKKi could be involved in the regulation of NF-kappaB activity in PC cells through a positive feedback loop. Supported by DOD Prostate Cancer Research Program DAMD 17-03-1-0522.

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Virginie Bottero, Vinay Tergaonkar, Masahito Ikawa, Qiutang Li & Inder M Verma. Laboratory of Genetics, The Salk Institute for Biological Studies, 10010, North Torrey Pines Road, La Jolla, CA 92037.

Antiapoptotic activity of NF κ B in tumors contributes to acquisition of resistance to chemotherapy. Degradation of I κ B is a seminal step in activation of NF κ B. The I κ B kinases, IKK1 and IKK2 have been implicated in both I κ B degradation and subsequent modifications of NF κ B. Using mouse embryo fibroblasts (MEFs) devoid of both IKK1 and 2 genes (IKK1/2^{-/-}), we document a novel I κ B degradation mechanism. We show that this degradation induced by chemotherapeutic agent, doxorubicin (DoxR), does not require the classical serine 32 and 36 phosphorylation or the PEST domain of I κ B α . Degradation of I κ B α is partially blocked by PI3kinase inhibitor LY294002 and is mediated by the proteasome. Free NF κ B generated by DoxR induced I κ B degradation in IKK1/2^{-/-} cells is able to activate chromatin based NF κ B reporter gene and expression of the endogenous target gene, I κ B α . These results also imply that modification of NF κ B by IKK1 or IKK2 either prior or subsequent to its release from I κ B is not essential for NF κ B mediated gene expression at least in response to DNA damage. Additionally, DoxR induced cell death in IKK1/2^{-/-} MEFs is enhanced by simultaneous inhibition of NF κ B activation by blocking the proteasome activity. These results reveal an additional pathway of activating NF κ B during the course of anti-cancer therapy and provide a mechanistic basis for the observation that proteasome inhibitors could be used as adjuvants in chemotherapy.

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4572 Effect of IKK-beta specific inhibitor PS1145 on NF-kappaB activity and apoptosis in prostate carcinoma cell lines.

Irina V. ■**Budunova**■, Alexander Yemelyanov, Alexander Gasparian, Lenny Dang, Jacqueline Pierce. *Northwestern University, Chicago, IL, National Cancer Research Center, Moscow, Russian Federation, Millenium Pharmaceuticals Inc., Cambridge, MA.*

Prostate cancer (PC) is the second leading cause of death among cancers in men. One of the contributing factors to high mortality rate from PC is the extreme resistance of malignant prostate cells to apoptosis induced by radio- and chemotherapy. Thus, the specific induction of apoptosis in PC cells could play a strategic role for PC treatment. One of the central mechanisms protecting cells from apoptotic death is mediated by NF-kappaB factors that control the expression of numerous anti-apoptotic genes. We and others showed previously that NF-kappaB transcription factor was constitutively active in PC cell lines and in human prostate tumors due to the up-regulated activity of IkappaB-kinases (IKK), mostly IKK-beta. In this work we investigated effect of a novel highly specific IKK-beta inhibitor PS1145 on constitutive and inducible NF-kappaB activity in human cell lines PC-3 and DU145 using Luciferase Assay with x5.kappaB-Luciferase reporter, EMSA, Northern blot analysis of expression of endogenous kappaB-responsive genes, Western blot analysis of IkappaBalpha phosphorylation, degradation and p65 nuclear translocation. Our studies revealed that PS1145 at the dose range 5-20 μ M efficiently inhibited both basal and induced by either TNF-alpha or LPS NF-kappaB activity in PC cells. PC3 and DU145 cells are known to be resistant to TNF-alpha-induced apoptosis partially due to the constitutively active NF-kappaB. We found that PS1145 significantly sensitized PC cell lines to TNF-alpha induced apoptosis. We observed the elevated PARP cleavage and caspase 3/7 activation when cells exposed to TNF-alpha were pretreated with PS1145. Currently we are evaluating the expression of kappaB-responsive genes as well as PC gene markers in prostate cells upon PS1145 treatment in vitro and in vivo. Supported by DOD prostate cancer research grants DAMD17-01-1-0015 and DAMD17-03-1-0522.

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